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Different types of multiple-synapse boutons in the cerebellar cortex between physically enriched and ataxic mutant mice

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Abstract

Experience-dependent synapse remodeling is associated with information storage in the nervous system. Neuronal synapses show alteration in various neurological and cognitive disorders in their structure and function. At the ultrastructural level, parallel fiber boutons contacting multiple spines of Purkinje cells in the cerebellar cortex are commonly observed in physiologically enriched animals as well as pathological ataxic mutants. However, the dendritic origin of those spines on parallel fiber multiple-synapse boutons (MSBs) has been poorly understood. Here, we investigated this issue by 3-dimensional ultrastructural analysis to determine synaptic connectivity of MSBs in both mice housed in physically enriched environment and cerebellar ataxic mutants. Our results demonstrated that environmental enrichment selectively induced MSBs to contact spines from the same parent dendrite, indicating focal strengthening of synapse through the simultaneous activation of two adjacent spines. In contrast, ataxic mutants displaying impaired motor coordination had significantly more MSBs involving spines originating from different neighboring dendrites compared to both wild-type and environmentally enriched animals, suggesting that compromising multiple synapse formation may lead to abnormal motor behavior in the mutant mice. These findings propose that environmental stimulation in normal animals mainly involves the refinement of preexisting synaptic networks, whereas pathological ataxic conditions may result from less-selective but compromising multiple synaptic formation. This study underscores that different types of multiple synapse boutons may have disparate effects on cerebellar synaptic transmission.

KEYWORDS

dendritic spine, environmental enrichment, parallel fiber, Purkinje cell, synapse

1 | INTRODUCTION

The excitatory synapses in the central nervous system are mostly composed of presynaptic axonal boutons and postsynaptic spines, which are tiny dendritic protrusions that display experience-dependent structural and functional remodeling (Harris & Stevens, 1988; Hering & Sheng, 2001; Yuste & Bonhoeffer, 2001). Neuronal synapses in the brain change their size, shape, number, and molecular composition in response to various physiological or pathological states (Luscher, Nicoll, Malenka, & Muller, 2000; Rakic, Bourgeois, & Goldman-Rakic, 1994). For many years, neuroscientists have focused on the synaptic plasticity

underlying information storage, as well as pathological synaptic alterations that may act as a causative factor in a subset of neurological and cognitive disorders.

Purkinje cells, a sole projection neuron in the cerebellar cortex, are innervated by two main excitatory inputs. Climbing fibers arising from the inferior olivary nucleus provide strong excitatory input to the primary dendrites of Purkinje cells, whereas parallel fibers (PF), bifurcated axons of granule cells, make weaker but numerous excitatory synapses to spines in higher order dendritic branches. As a form of physiological learning, motor skill learning requires repetitive practice (Kleim, Vij, Ballard, & Greenough, 1997) and proper cerebellar function (Ito, 2000;

Thach, 1998; Thach, Goodkin, & Keating, 1992). A growing body of literature has shown that motor skill training induces new synapse formation in the cerebellar cortex selectively between PF boutons and dendritic spines of Purkinje cells (Anderson, Alcantara, & Greenough, 1996; Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Federmeier, Kleim, & Greenough, 2002; Kim et al., 2002; Kleim et al., 1998; Lee et al., 2013a). Similarly, adult rats trained with acrobatic motor skill tasks had more dendritic spines in the distal dendrites of Purkinje cells that are mainly innervated by PFs (Lee, Jung, Arai, Imoto, & Rhyu, 2007). At the ultrastructural level, PF-to-Purkinje cell synapses can be categorized into three types by their discrete contact features (Kim et al., 2002; Lee et al., 2013a). Single-synapse boutons (SSBs) contacting one postsynaptic spine are most prominently found, although multiple-synapse boutons (MSBs) involving two dendritic spines are also frequently observed. In contrast, multiple-synapse spines that make synapses with two PF boutons are very rarely detected. Quantitative stereological analysis of electron microscopic images has shown that training rats with complex motor skill tasks increased the number of PF MSBs per Purkinje cell (Federmeier et al., 2002; Kim et al., 2002; Lee et al., 2013a). Furthermore, PF MSBs induced by motor skill learning were revealed to contact two spines from the same dendritic origin (Lee et al., 2013a), which supports the enhancement of local synaptic efficacy. However, it is unclear whether those same-dendrite MSBs can be produced in different species as well as more natural behavioral stimulation, such as environmental enrichment (EE).

The EE is one of the most widely used behavioral paradigms to study experience-dependent synaptic plasticity by boosting animal's explorative behavior, curiosity, and attention. Under this condition, animals are exposed to complex physical and social surroundings, including running wheels, tunnels, mazes, and toys. An essential component of the EE procedure is sustained physical exercise and motor skill acquisition. A number of studies have shown that hippocampal or cortical neurons in rodents raised in an enriched environment have more synapses per neuron, higher spine density, and increased dendritic complexity compared to control animals (Leuner & Gould, 2010; Nithianantharajah & Hannan, 2006). Similarly, a recent study demonstrated that spine density and size (head diameter, length, and area) in the distal dendrites of Purkinje cells were also higher in the enriched animals (De Bartolo, Florenzano, Burello, Gelfo, & Petrosini, 2015). Despite strong evidence of PF MSB formation following motor skill training, the EE effect on MSB formation in the cerebellar cortex has not been described.

Ataxia is a neuropathological symptom that is characterized by the lack of motor coordination. The tottering (tg/tg) mouse is a mild ataxic model that carries an autosomal recessive mutation in the gene coding for the Cav2.1 (P/Q-type) Ca^{2+} channel $\alpha 1\text{A}$ subunit and exhibits episodic motor attacks, absence seizures, and paroxysmal motor defects (Fletcher et al., 1996). In the cerebellar cortex, the molecular layer volume was specifically decreased in adult tottering mice compared to age-matched wild-type (WT) animals, although the volume of granule cell layer were not affected (Isaacs & Abbott, 1995). Ultrastructural investigation has found that individual PF boutons in tottering mice often make multiple synapses with Purkinje cell dendritic spines (Rhyu, Abbott, Walker, & Sotelo, 1999). These findings suggest that impaired

motor coordination in tottering mutant mice results from aberrant circuit formation of PF-to-Purkinje cell synapses in the cerebellar cortex. Taken together, it is evident that PF MSB formation is commonly induced in both physiological motor skill learning and pathological ataxic mutation (Gray, 1959). However, the dendritic origin of PF MSB-contacting spines has not been completely understood in those conditions. Here we studied this issue by 3-dimensional ultrastructural analysis to determine synaptic connectivity of MSBs in both mice housed in physically enriched environment and cerebellar ataxic mutants.

2 | MATERIALS AND METHODS

2.1 | Physiological model: Environmental enrichment

C57BL/6 mice (3-weeks-old) were randomly allocated to either standard (20-cm $\times$ 15-cm $\times$ 13-cm, $n = 5$) laboratory plastic cages or an enlarged EE special cage (40-cm $\times$ 25-cm $\times$ 28-cm, $n = 15$) for 4 weeks (Figure 1a). Animals were maintained at room temperature and humidity with a 12-h light: 12-h dark cycle. The EE group was housed with wheels, tunnels, plastic tires, platforms, and wooden toy houses, according to the procedure specified in the previous report (De Bartolo et al., 2015) with a slight modification (Figure 1b). All animal experiments are in compliance with the guidelines for Korea University Institutional Animal Care & Use Committee (KUIACUC-2011-222).

2.2 | Pathological model: Tottering mutant mice

Homozygous tottering (tg/tg) mice and C57BL/6J WT mice ($n = 5$ per group) were housed in standard mouse cages and under conventional laboratory conditions: constant temperature and humidity control with a 12-h light: 12-h dark cycle. The tottering mice exhibited abnormal wobbly gaits around 3 to 4 weeks of age. This obvious behavioral phenotype and an additional genetic marker, oligosyndactyly (Os), were used to identify the genotype of individual animals (Rhyu et al., 1999).

2.3 | Serial section electron microscopy

All animals were sacrificed immediately after behavioral experiments. Mice were anesthetized and perfused with normal saline followed by 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The cerebella were dissected out and stored in the same fixative overnight at 4°C. The small blocks of brain were post-fixed in 2% Osmium tetroxide solution for 2 hr, and en bloc stained with 0.2% aqueous uranyl acetate for 1 hr. After dehydration through an ascending ethanol series, cerebellar tissues were embedded in epoxy resin mixture. Polymerized blocks were fine trimmed into the paramedian lobule. Sixty to seventy serial ultrathin sections (thickness = 70 nm) were cut from the middle of the molecular layer using a diamond knife on a Leica EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections were mounted on Formvar-coated one-hole grids and subsequently post-stained with heavy metals. Serial images (3,000–6,000 $\times$) were randomly obtained from three different areas of the paramedian lobule per animal using a transmission electron microscope (Hitachi H-7650, Hitachi, Tokyo, Japan) at the accelerating voltage of 80 kV.

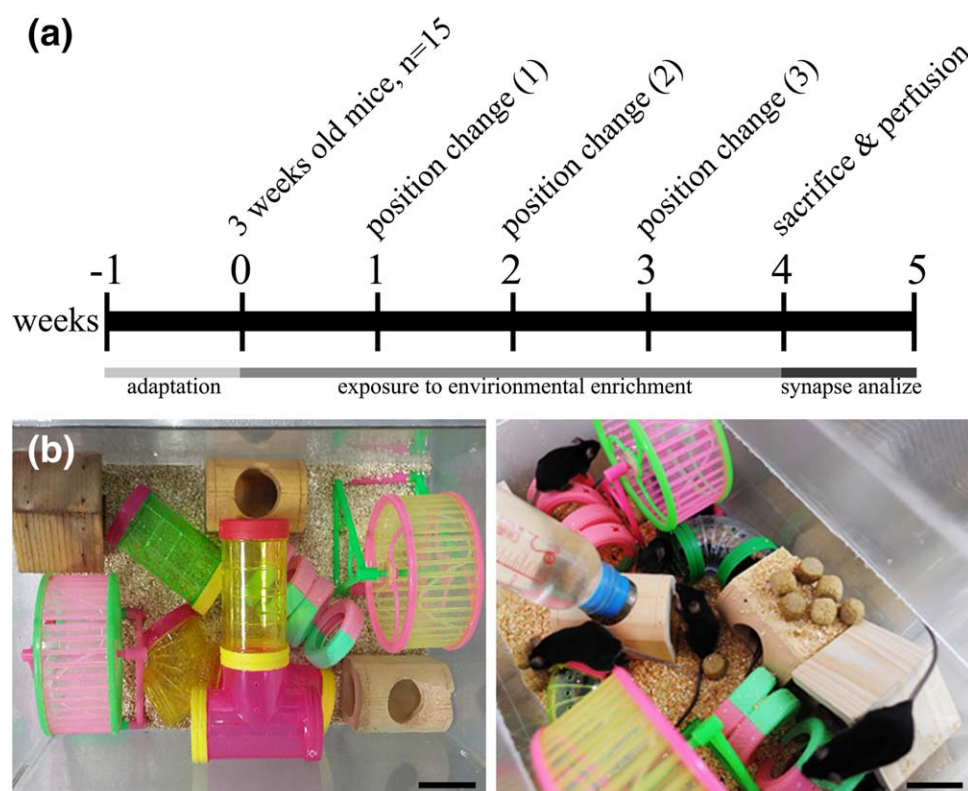


FIGURE 1 Schematic of enriched housing condition. (a) Experimental time lines. (b) The enriched environment consisted of an enlarged cage with running wheels, tunnels, plastic tires, platforms, and wooden toy houses to increase explorative activity and motor skill acquisition. All toy positions were changed once a week during the experimental period. Scale bar = 5 cm [Color figure can be viewed at wileyonlinelibrary.com]

2.4 | Stereological analysis

All ultrastructural analyses were performed blind to experimental conditions. For counting the number of synapses per unit volume, we used the physical disector technique, which is an efficient and unbiased stereological method to estimate object density (Gundersen et al., 1988). For each animal, three different z-stacks of twenty serial images obtained from the paramedian lobule were aligned using Reconstruct® software using disector module (Figure 2a, b). PF-to-Purkinje cell synapses within the counting frame were identified based on the distribution of synaptic vesicles and existence of clear postsynaptic density (PSD). PF boutons mostly had small clusters of synaptic vesicles, whereas much larger climbing fiber boutons were filled up with densely packed synaptic vesicles (Cesa, Morando, & Strata, 2003). PF-to-Purkinje cell synapses were classified into SSBs contacting one spine and MSBs contacting more than two spines (SSB: Control, $n = 717$; EE, $n = 668$; WT, $n = 859$; tg/tg, $n = 676$) (MSB: Control, $n = 98$; EE, $n = 259$; WT, $n = 115$; tg/tg, $n = 292$). The number of synapse per unit volume (S_v) was calculated based on the routine equation.

2.5 | Three-dimensional tracing of MSBs

For each MSB, thirty to forty serial images were recorded to identify the dendritic origin of spines on them. Individual spines on MSBs were marked using different colors and tracing was continued until they reached their parent dendrites (Figure 3a). In each animal, each MSB

was further figured out to be same-dendrite MSBs (sdMSBs) or different-dendrite MSBs (ddMSBs) (Figure 3b,c) (MSB: Control, $n = 20$; EE, $n = 25$; WT, $n = 27$; tg/tg, $n = 31$). The number of sdMSBs and ddMSBs was expressed as a percentage of total MSBs.

3 | RESULTS

3.1 | Synapse in the cerebellar cortex of enriched mice

Motor skill training of adult rats induces new synapse formation between presynaptic PF boutons and dendritic spines of Purkinje cells in the cerebellar cortex. Indeed, we and others have consistently observed an increase in the number of SSBs and MSBs in the rat cerebellar cortex, as well as layer V of the primary motor cortex, following complex acrobatic training (Anderson et al., 1996; Black et al., 1990; Federmeier et al., 2002; Jones, Chu, Grande, & Gregory, 1999; Kleim, Lussnig, Schwarz, Comery, & Greenough, 1996; Lee et al., 2007). To test whether EE, a more natural brain stimulating paradigm involving motor skill acquisition, can also produce PF MSBs in the cerebellar cortex of mice, we estimated synaptic density in a series of consecutive electron microscopic images using the physical disector method. Consistent with a recent study (De Bartolo et al., 2015), we found a significant increase in the number of PF-to-Purkinje cell synapses per unit volume in enriched mice compared to control animals (Control, $0.63 \pm 0.02/\mu\text{m}^3$; EE, $0.83 \pm 0.02/\mu\text{m}^3$; Student *t*-test, $p < .0001$, $n = 5$

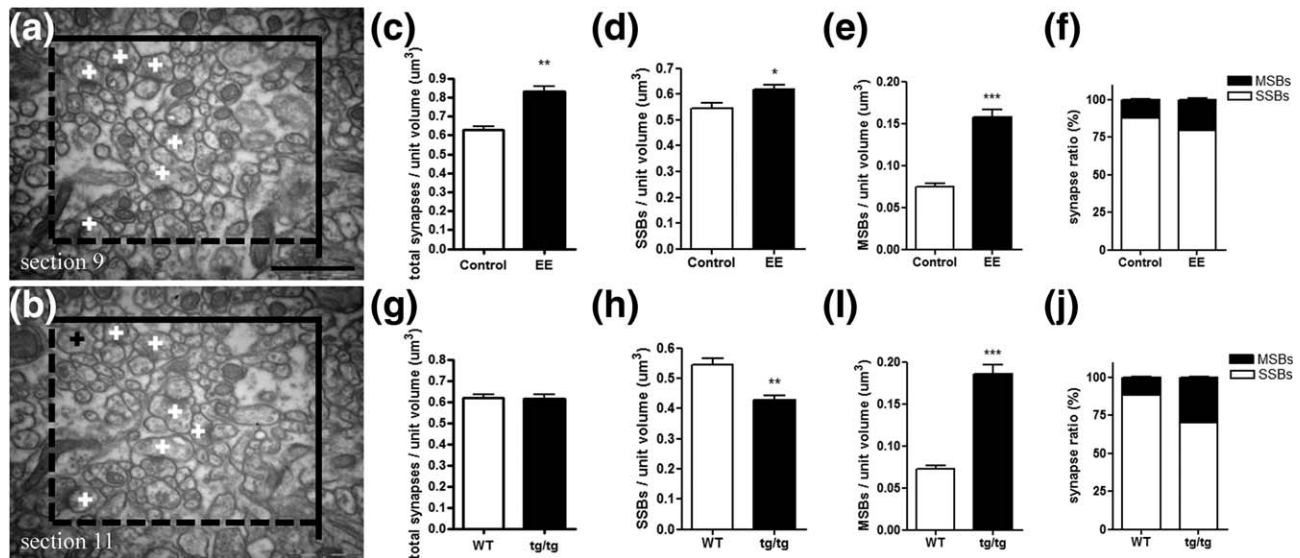


FIGURE 2 Ultrastructural changes in parallel fiber-to-Purkinje cell synapses in enriched and ataxic mutant mice. (a and b) The physical disector was used to estimate synaptic density. Synapses identified within a counting frame on section 9 (white cross mark) were examined on the consecutive images and counted when their PSD completely disappeared (black cross mark on section 11). (c–j) Quantification of the number of total synapses, SSBs and MSBs in a unit volume in enriched (c–e) and tottering mice (g–i). The average percentage of SSBs and MSBs were also presented on the right (f and j) (enriched mice: SSBs, $p < .05$; MSBs, $p < .0001$; tottering mice: SSBs, $p < .01$; MSBs, $p < .0001$; Student t -test). Scale bar = $1 \mu\text{m}$. Data are means $\pm$ SEM

animals per group) (Figure 2c). PF-to-Purkinje cell synapses were further classified as SSBs contacting only one spine and MSBs contacting more than two spines. Unbiased stereological analysis revealed that enriched mice had significantly more MSBs (Control, $0.08 \pm 0.005/\mu\text{m}^3$; EE, $0.16 \pm 0.007/\mu\text{m}^3$; Student t -test, $p < .0001$, $n = 5$ animals per group) and SSBs (Control, $0.55 \pm 0.02/\mu\text{m}^3$; EE, $0.67 \pm 0.018/\mu\text{m}^3$; Student t -test, $p = .0286$, $n = 5$ animals per group) than sedentary animals (Figure 2d,e). The average percentage of SSBs and MSBs in control mice was $88.05 \pm 0.38\%$ and $11.95 \pm 0.38\%$, respectively. In contrast, the percentage of MSBs in the EE condition was approximately 1.7-fold higher than control group (EE condition: SSBs, $79.59 \pm 0.59\%$; MSBs, $20.41 \pm 0.59\%$) (Figure 2f). These results demonstrate that the EE also induces MSBs as well as SSBs in the cerebellar paramedian lobule of mice.

3.2 | Synapse in the cerebellar cortex of ataxic mutants

Subsequently, we investigated whether synaptic density and the composition of PF-to-Purkinje cell synapses were affected in the cerebellar cortex of tottering mutant mice. Again, we exploited the disector counting method with serial electron micrographs. No difference in synapse number between tottering and wild types was observed (WT, $0.62 \pm 0.02/\mu\text{m}^3$; tg/tg, $0.61 \pm 0.02/\mu\text{m}^3$; Student t -test, $p = .71$, $n = 5$ per group) (Figure 2g). However, further analysis showed that the tottering mice had significantly more MSBs than WT animals (WT, $0.07 \pm 0.003/\mu\text{m}^3$; tg/tg, $0.19 \pm 0.009/\mu\text{m}^3$; Student t -test, $p < .0001$; Figure 2i), whereas a substantial decrease in the number of SSBs were observed in tottering mice (WT, $0.55 \pm 0.02/\mu\text{m}^3$; tg/tg, $0.43 \pm 0.01/\mu\text{m}^3$; Student t -test, $p < .01$; Figure 2h), suggesting reorganization of synaptic circuitry in the mutants with CaV2.1 channelopathy.

Accordingly, average percentages of SSBs and MSBs were obviously different between groups (SSBs: WT, $88.14 \pm 0.68\%$; tg/tg, $70.02 \pm 0.71\%$; MSBs: WT, $11.86 \pm 0.68\%$; tg/tg, $29.98 \pm 0.71\%$) (Figure 2j). These findings demonstrate that MSB formation is also promoted in the cerebellar cortex under neuropathological condition.

3.3 | Three-dimensional connectivity of MSBs in physiological and pathological conditions

Our results underscore that MSBs can be formed under both physiological and pathological conditions. However, functional roles of MSBs in both conditions would be different depending on the dendritic origin of MSB-contacting spines (Lee et al., 2013a): if two spines on MSBs originate from the same dendrite (sdMSBs; Figure 3b), local synaptic strengthening would be expected by simultaneous activation of both spines. In contrast, if MSB-contacting spines arise from different dendrites (ddMSBs; Figure 3c), it would be predicted that synapses have recruited an extra spine from a neighboring dendrite. To address this issue, we investigated the dendritic origin of MSB-contacting spines in the enriched and tottering mice using three-dimensional tracings of serial electron micrographs (Figure 3a). We found that the EE group had a significantly larger portion of sdMSBs than control (Control, $76 \pm 4.12\%$; EE, $83 \pm 4.87\%$; Student t -test, $p = .0329$, $n = 20$ –25 MSBs per group; Figure 3d), indicating an enhanced local synaptic efficacy. On the contrary, ddMSBs were more frequently observed in tottering mice (WT, $26 \pm 2.64\%$; tg/tg, $51 \pm 4.17\%$; Student t -test, $p < .0001$, $n = 27$ –31 MSBs per group; Figure 3e), suggesting a less selective but compromising recruitment of neighboring spines. Together, these findings suggest that MSB formation between physiological and pathological conditions is achieved with different purposes in the cerebellar cortex.

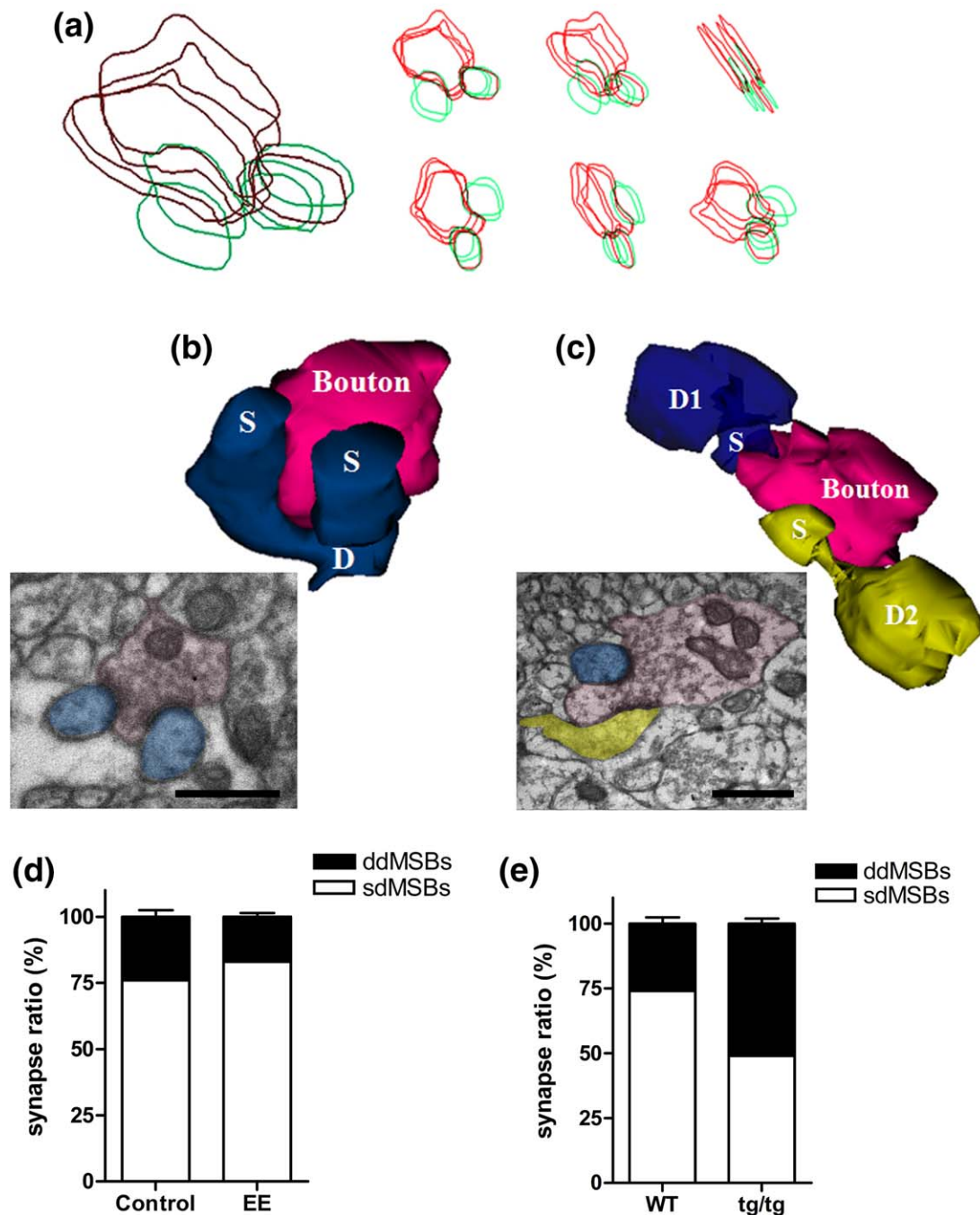


FIGURE 3 Three-dimensional tracing and dendritic origin of MSB-contacting spines. The Reconstruct[®] software was used for the analysis (a). Brown and red colors indicate presynaptic axonal boutons and green color denotes dendritic spines, respectively. MSBs were classified as same dendrite MSBs (sdMSBs) (b) or different dendrite MSBs (ddMSBs) (c) according to the origins of spine pairs. (d and e) Quantification of the average percentage of sdMSBs and ddMSBs. Enriched mice had more sdMSBs, whereas ddMSBs were more predominantly found in tottering mice. S, spine; D, dendrite; Bouton, parallel fiber bouton; Scale bar, 0.5 μ m. Data are means $\pm$ SEM [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

This study revealed that EE induced PF MSBs that primarily contacted spine pairs of same dendritic origin, which implies local synaptic strengthening. In contrast, MSB spines in tottering mice more frequently originated from different nearby dendritic segments, reflecting that less-selective network formation would be a cause of behavioral

symptoms. These results suggest that different types of MSB formation in physiological and pathological conditions may have disparate functional roles in information processing in the cerebellar cortex.

MSBs have been found in multiple brain regions, including cerebellum, primary motor cortex, visual cortex, and hippocampus, following various behavioral training (Federmeier et al., 2002; Geinisman, Berry, Disterhoft, Power, & Van der Zee, 2001; Jones et al., 1999; Jones,

Klintsova, Kilman, Sirevaag, & Greenough, 1997; Kim et al., 2002; Lee et al., 2013a), estrogen treatment (Woolley, Wenzel, & Schwartzkroin, 1996; Yankova, Hart, & Woolley, 2001), and the induction of long-term potentiation (LTP) (Fiala, Allwardt, & Harris, 2002; Medvedev et al., 2014; Toni, Buchs, Nikonenko, Bron, & Muller, 1999). These findings suggest that MSB formation may be widely associated with storing neural information in physiological conditions. Nonetheless, this idea has been somewhat confused by the observations that MSBs are also formed in pathological conditions, including motor discoordination, anoxia-hypoglycemia, and chronic alcohol consumption (Jourdain, Nikonenko, Alberi, & Muller, 2002; Rhyu et al., 1999; Tavares, Paula-Barbosa, & Verwer, 1987). Similarly, our present data showed that MSBs in two-dimensional EM images are commonly observed in both physiologically stimulating conditions and pathological ataxic mutants. However, conventional electron microscopy using ultrathin section thickness (typically 50–100 nanometers) provides only fragmented, two-dimensional profiles of MSBs. Thus, it is difficult to understand the functional implications of MSBs without identifying dendritic origin of postsynaptic spines on them. To resolve this, we employed serial section electron microscopy (Harris & Stevens, 1988; Lee et al., 2013a; Xu-Friedman, Harris, & Regehr, 2001) and compared three-dimensional synaptic connectivity of MSBs between physiological and pathological conditions. Our results revealed that each condition employs different types of MSB formation in the cerebellar paramedian lobule. In the EE group, MSB-contacting spines mainly originated from the same parent dendrites. In contrast, MSBs in tottering mice more frequently contacted spines arising from neighboring dendritic segments compared to WT animals. We speculate that physiological learning may involve the fine-tuning of preexisting synaptic circuits by engaging an additional spine from the same dendrite. In support of this idea, a recent study has proposed that sdMSBs induced by motor skill training may serve as ultrastructural correlates of persistent local synaptic strengthening because simultaneous activation of two adjacent spines on the same dendrite would produce a greater net response (Lee et al., 2013a). Indeed, sdMSBs have been induced in area CA1 of rat hippocampal slices as well as the dentate gyrus of awake adult rats after LTP stimulation (Fiala et al., 2002; Medvedev et al., 2014; Toni et al., 1999).

The selective formation of sdMSBs in EE animals apparently support the enhanced efficacy of excitatory PF synapses onto Purkinje cells, favoring of LTP mechanism. However, it should be noted that EE used in this study is composed of various factors such as learning, exploration, motor activity, familiarity, and motivation that could affect several brain areas as well as multiple sites in the cerebellum. Therefore, it is plausible that this complex behavioral condition would induce a concerted recruitment of LTP, LTD, and other types of cellular plasticity mechanisms at the cellular and circuit levels. In line with this, it would be of interest to mention that LTP and LTD at PF to Purkinje cell synapses are both presynaptically and postsynaptically expressed and fully reversible in cerebellar *in vitro* slice system. Conversely, different cerebellar motor learning paradigms such as vestibule-ocular adaptation and associative eyeblink conditioning have been shown to be asymmetric and not equally reversible. This discrepancy between *in vitro* and *in vivo* conditions may result from the unequal and selective

recruitment of presynaptic and postsynaptic plasticity mechanisms during *in vivo* learning conditions although it remains unclear under what circumstances they are recruited and why both presynaptic and postsynaptic forms of LTD and LTP exist (Titley & Hansel, 2016).

Conversely, we found more ddMSBs in tottering mice. In the mechanistic view, we propose that more ddMSBs in ataxic mutants could be the cause of motor dysfunction. In support, a previous study showed PF MSBs before the onset of behavioral symptoms (postnatal 2–3 weeks) (Rhyu et al., 1999). Considering the cerebellar granule cells start a tangential movement after birth and then make a progressive synaptic circuits with Purkinje cell spines, it is plausible that ataxic symptom is not clearly observed in early juvenile tottering mice as reported (Fletcher et al., 1996). Furthermore, homozygous leaner mice exhibiting earlier onset of cerebellar ataxia (postnatal days 10–12) compared to tottering mice had MSBs involving more numbers of Purkinje cell spines ranging from 2 to 9 spines per PF bouton (unpublished data). Collectively, these data suggest that more ddMSBs in these mutant mice may be the cause, but not product, of the ataxic symptoms.

Because electrophysiological recording in the mutant cerebellar slices revealed the reduced amplitude of excitatory postsynaptic current in PF-to-Purkinje cell synapses (Oda et al., 2010), one possible explanation of ddMSB formation during the development of mutant animals is that mutant PFs may recruit their nearby postsynaptic partners as a compensatory mechanism, although this less-selective compensatory synaptic recruitment is not sufficient to overcome their motor behavioral symptoms. In line with this notion, it has been reported that chronic inactivity homeostatically up-regulates the number of postsynaptic contacts at hippocampal mossy fiber-CA3 synapses (Lee et al., 2013b). Furthermore, an allele of tottering, rolling mouse Nagoya showed abnormalities in Purkinje cell dendritic spines that projected in irregular direction, which might contribute to the formation of less-selective ddMSBs (Oda et al., 2010). These findings may indicate that some neurological disorders, including cerebellar ataxia, are caused by anatomical connectopathies with stereotyped defects that impair the specificity of synaptic circuitry.

It should be noted that spines on ddMSB can actually be on the same dendritic branch with greater distance or on neighboring dendritic branches arising from the same neuron. Serial EM image stacks covering a much larger field of view are necessary to address this question. Nonetheless, this will not change the main point of our study that more ddMSBs in the ataxic mutant mice may represent abnormal and diffusive synaptic connectivity, which is clearly different from the strengthening of local synaptic efficacy via sdMSBs following environmental enrichment.

In summary, this study demonstrates that MSBs observed in physiological and pathological conditions have qualitatively different synaptic connectivity. A selective increase in the number of sdMSBs under EE may enhance local synaptic efficacy in a subset of PF-to-Purkinje cell synapses, whereas ddMSBs in tottering mice may represent a compensatory mechanism to augment neurotransmission, although it is not sufficient to recover behavioral impairments. Therefore, MSBs in both conditions may be formed with disparate purposes in neural

information processing. It would be of interest to investigate if EE exposure of tottering mice in the early developmental period can restore ultrastructural and behavioral phenotypes observed in their adulthood, and examine if there is a change in balance between excitatory and inhibitory synapses in these animals, which has been raised as a critical factor for some neurological disorders. Further studies are warranted to better understand the functional roles of different types of multiple-synapse formation in the brain.

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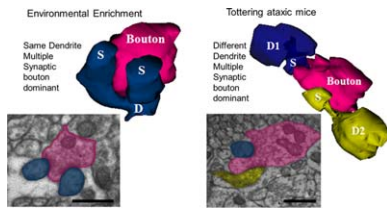
REFERENCES

- Anderson, B. J., Alcantara, A. A., & Greenough, W. T. (1996). Motor-skill learning: changes in synaptic organization of the rat cerebellar cortex. *Neurobiology of Learning and Memory*, 66(2), 221–229.
- Black, J. E., Isaacs, K. R., Anderson, B. J., Alcantara, A. A., & Greenough, W. T. (1990). Learning causes synaptogenesis, whereas motor activity causes angiogenesis, in cerebellar cortex of adult rats. *Proceedings of the National Academy of Sciences of the United States of America*, 87(14), 5568–5572.
- Cesa, R., Morando, L., & Strata, P. (2003). Glutamate receptor delta2 subunit in activity-dependent heterologous synaptic competition. *The Journal of Neuroscience*, 23(6), 2363–2370.
- De Bartolo, P., Florenzano, F., Burello, L., Gelfo, F., & Petrosini, L. (2015). Activity-dependent structural plasticity of Purkinje cell spines in cerebellar vermis and hemisphere. *Brain Structure & Function*, 220(5), 2895–2904.
- Federmeier, K. D., Kleim, J. A., & Greenough, W. T. (2002). Learning-induced multiple synapse formation in rat cerebellar cortex. *Neuroscience Letters*, 332(3), 180–184.
- Fiala, J. C., Allwardt, B., & Harris, K. M. (2002). Dendritic spines do not split during hippocampal LTP or maturation. *Nature Neuroscience*, 5(4), 297–298.
- Fletcher, C. F., Lutz, C. M., O'Sullivan, T. N., Shaughnessy, J. D., Jr., Hawkes, R., Frankel, W. N., ... Jenkins, N. A. (1996). Absence epilepsy in tottering mutant mice is associated with calcium channel defects. *Cell*, 87(4), 607–617.
- Geinisman, Y., Berry, R. W., Disterhoft, J. F., Power, J. M., & Van der Zee, E. A. (2001). Associative learning elicits the formation of multiple-synapse boutons. *The Journal of Neuroscience*, 21(15), 5568–5573.
- Gray, E. G. (1959). Axi-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *Journal of Anatomy*, 93, 420–433.
- Gundersen, H. J., Bagger, P., Bendtsen, T. F., Evans, S. M., Korbo, L., Marcussen, N., ... Pakkenberg, B. (1988). The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Acta Pathologica, Microbiologica, Et Immunologica Scandinavica*, 96(10), 857–881.
- Harris, K. M., & Stevens, J. K. (1988). Dendritic spines of rat cerebellar Purkinje cells: serial electron microscopy with reference to their biophysical characteristics. *The Journal of Neuroscience*, 8(12), 4455–4469.
- Hering, H., & Sheng, M. (2001). Dendritic spines: structure, dynamics and regulation. *Nature Reviews Neuroscience*, 2(12), 880–888.
- Isaacs, K. R., & Abbott, L. C. (1995). Cerebellar volume decreases in the tottering mouse are specific to the molecular layer. *Brain Research Bulletin*, 36(3), 309–314.
- Ito, M. (2000). Mechanisms of motor learning in the cerebellum. *Brain Research*, 886(1–2), 237–245.
- Jones, T. A., Chu, C. J., Grande, L. A., & Gregory, A. D. (1999). Motor skills training enhances lesion-induced structural plasticity in the motor cortex of adult rats. *The Journal of Neuroscience*, 19(22), 10153–10163.
- Jones, T. A., Klintsova, A. Y., Kilman, V. L., Sirevaag, A. M., & Greenough, W. T. (1997). Induction of multiple synapses by experience in the visual cortex of adult rats. *Neurobiology of Learning and Memory*, 68(1), 13–20.
- Jourdain, P., Nikonenko, I., Alberi, S., & Muller, D. (2002). Remodeling of hippocampal synaptic networks by a brief anoxia-hypoglycemia. *The Journal of Neuroscience*, 22(8), 3108–3116.
- Kim, H. T., Kim, I. H., Lee, K. J., Lee, J. R., Park, S. K., Chun, Y. H., ... Rhyu, I. J. (2002). Specific plasticity of parallel fiber/Purkinje cell spine synapses by motor skill learning. *Neuroreport*, 13(13), 1607–1610.
- Kleim, J. A., Lussnig, E., Schwarz, E. R., Comery, T. A., & Greenough, W. T. (1996). Synaptogenesis and Fos expression in the motor cortex of the adult rat after motor skill learning. *The Journal of Neuroscience*, 16(14), 4529–4535.
- Kleim, J. A., Swain, R. A., Armstrong, K. A., Napper, R. M., Jones, T. A., & Greenough, W. T. (1998). Selective synaptic plasticity within the cerebellar cortex following complex motor skill learning. *Neurobiology of Learning and Memory*, 69(3), 274–289.
- Kleim, J. A., Vij, K., Ballard, D. H., & Greenough, W. T. (1997). Learning-dependent synaptic modifications in the cerebellar cortex of the adult rat persist for at least four weeks. *The Journal of Neuroscience*, 17(2), 717–721.
- Lee, K. J., Jung, J. G., Arii, T., Imoto, K., & Rhyu, I. J. (2007). Morphological changes in dendritic spines of Purkinje cells associated with motor learning. *Neurobiology of Learning and Memory*, 88(4), 445–450.
- Lee, K. J., Park, I. S., Kim, H., Greenough, W. T., Pak, D. T., & Rhyu, I. J. (2013a). Motor skill training induces coordinated strengthening and weakening between neighboring synapses. *Journal of Neuroscience*, 33(23), 9794–9799.
- Lee, K. J., Queenan, B. N., Rozeboom, A. M., Bellmore, R., Lim, S. T., Vicini, S., & Pak, D. T. (2013b). Mossy fiber-CA3 synapses mediate homeostatic plasticity in mature hippocampal neurons. *Neuron*, 77(1), 99–114.
- Leuner, B., & Gould, E. (2010). Structural plasticity and hippocampal function. *Annual Review of Psychology*, 61, 111–140. C1–3.
- Luscher, C., Nicoll, R. A., Malenka, R. C., & Muller, D. (2000). Synaptic plasticity and dynamic modulation of the postsynaptic membrane. *Nature Neuroscience*, 3(6), 545–550.
- Medvedev, N. I., Dallerac, G., Popov, V. I., Rodriguez Arellano, J. J., Davies, H. A., Kraev, I. V., ... Stewart, M. G. (2014). Multiple spine boutons are formed after long-lasting LTP in the awake rat. *Brain Structure & Function*, 219(1), 407–414.
- Nithianantharajah, J., & Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews Neuroscience*, 7(9), 697–709.

- Oda, S. I., Lee, K. J., Arai, T., Imoto, K., Hyun, B. H., Park, I. S., ... Rhyu, I. J. (2010). Differential regulation of Purkinje cell dendritic spines in rolling mouse Nagoya (tg/tg), P/Q type calcium channel ($\alpha 1(A)/Ca(v)2.1$) mutant. *Anatomy & Cell Biology*, 43(3), 211–217.
- Rakic, P., Bourgeois, J. P., & Goldman-Rakic, P. S. (1994). Synaptic development of the cerebral cortex: implications for learning, memory, and mental illness. *Progress in Brain Research*, 102, 227–243.
- Rhyu, I. J., Abbott, L. C., Walker, D. B., & Sotelo, C. (1999). An ultrastructural study of granule cell/Purkinje cell synapses in tottering (tg/tg), leaner (tg(la)/tg(la)) and compound heterozygous tottering/leaner (tg/tg(la)) mice. *Neuroscience*, 90(3), 717–728.
- Tavares, M. A., Paula-Barbosa, M. M., & Verwer, R. W. (1987). Synapses of the cerebellar cortex molecular layer after chronic alcohol consumption. *Alcohol (Fayetteville, N.Y.)*, 4(2), 109–116.
- Thach, W. T. (1998). What is the role of the cerebellum in motor learning and cognition?. *Trends in Cognitive Sciences*, 2(9), 331–337.
- Thach, W. T., Goodkin, H. P., & Keating, J. G. (1992). The cerebellum and the adaptive coordination of movement. *Annual Review of Neuroscience*, 15, 403–442.
- Titely, H. K., & Hansel, C. (2016). Asymmetries in cerebellar plasticity and motor learning. *Cerebellum (London, England)*, 15(2), 87–92.
- Toni, N., Buchs, P. A., Nikonenko, I., Bron, C. R., & Muller, D. (1999). LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. *Nature*, 402(6760), 421–425.
- Woolley, C. S., Wenzel, H. J., & Schwartzkroin, P. A. (1996). Estradiol increases the frequency of multiple synapse boutons in the hippocampal CA1 region of the adult female rat. *The Journal of Comparative Neurology*, 373(1), 108–117.
- Xu-Friedman, M. A., Harris, K. M., & Regehr, W. G. (2001). Three-dimensional comparison of ultrastructural characteristics at depressing and facilitating synapses onto cerebellar Purkinje cells. *The Journal of Neuroscience*, 21(17), 6666–6672.
- Yankova, M., Hart, S. A., & Woolley, C. S. (2001). Estrogen increases synaptic connectivity between single presynaptic inputs and multiple postsynaptic CA1 pyramidal cells: A serial electron-microscopic study. *Proceedings of the National Academy of Sciences of the United States of America*, 98(6), 3525–3530.
- Yuste, R., & Bonhoeffer, T. (2001). Morphological changes in dendritic spines associated with long-term synaptic plasticity. *Annual Review of Neuroscience*, 24, 1071–1089.

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Multiple-synapse boutons (MSB) are observed in the cerebellum of both environmentally enriched (EE) and tottering ataxic mutant mice. MSBs from EE mice contact two spines originating from the same dendritic segments, but MSBs from ataxic mutants contact two spines of different dendritic origins.